RELATIONSHIP IN MICE BETWEEN INHIBITION OF SALIVATION AND TOXICITY OF CIGARETTE SMOKE*

by

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Acute toxicity and salivary inhibition in mice - Van Dongen

Mr. Chairman, Members of the Society:

We reported previously on a non-traumatic method for the quantitative measurement of salivation in minimally restrained unanesthetized mice.

Narrow cotton fabric strips, pretreated with cresol-red indicator, are inserted into the mouth by means of an oral wire bit to absorb saliva. The length of the diffused colored band, visible after a given time interval, is measured in mm. and indicates the amount of saliva excreted. Immobilization of the mice for measuring salivation is achieved by fitting a "collar" around the neck of the animals and inserting the collar into a "stanchion" during measurements. Such an arrangement permits the simultaneous study of salivation in a large "herd" of mice.

Slide I shows a mouse in a stanchion by its permanently worn collar. The mouse bites on a wire bit. A narrow cotton strip, attached to the bit turns then from yellow to purple upon exposure to saliva.

The next slide shows that a large number of mice can be studied simultaneously. Significant differences in salivary performance exist among various strains of mice. High-salivating C57BL mice. Low-salivating AKR mice. All supplied by the Jackson Laboratories in Bar Harbor, Maine.

The above described salivation method in mice has been applied previously to study the effects of various physiological factors, such as sex and strains on salivary performance. The method also proved to be a useful tool as a bio-assay for cholinergic and anti-cholinergic drug substances. Recent pilot experiments, moreover, showed that the exposure of mice to cigarette smoke reduced their salivation, and that this effect is less pronounced while only the gasphase of such smoke is employed. These findings suggest the possibility of a relationship between salivary performance and toxicity of cigarette smokes.

The specific objectives of this study are: (1) to establish the acute toxicities of whole smokes from these two brands of cigarettes; (2) to confirm or disprove the inhibitory action of whole tobacco smokes on salivary performance in a dose-related fashion; (3) to examine any differential inhibitory action between the whole smokes of two cigarette brands; (4) to examine some experimental conditions with the objective of obtaining the salivation inhibitory actions with higher resolution.

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To perform both types of experiments, a large herd of male C57BL mice was available. The animals were approximately 24 weeks old, had been housed for some time six to a cage with their collars on. They also had been adapted previously to the salivation procedure and were all in good health.

In the salivation experiment, salivary performance of mice was determined five times with a one-hour interval between measurements. The first and second measurements served to establish the control salivary performance of individual mice during that particular day. A ten-minute smoke exposure was applied immediately prior to the third measurement. The fourth and fifth measurements were performed to examine any after-effects to treatments. During the time intervals between any two measurements, the mice were returned to their cages which were supplied with adequate food and water. Salivary performance in mice was determined according to the above mentioned standard procedures.

Smoke exposures were applied with the Walton-reverse smoking machine. The conditions in the smoking machine were standardized to 35 ml. puffs of two seconds' duration taken at intervals of 58 seconds. The smoke would persist for 15 seconds in the inhalation chamber and then be expulsed in 3 seconds followed by a fresh-air purge for 40 seconds. Ten puffs were studied in each case, using the first 5 puffs of each of two sets of cigarettes, smoked without interruption in the one puff per minute cycle.

We applied any one of the following ten treatments, namely, no machine exposure, machine exposure with air, or any one of four smoke dilutions from whole smokes of brand A or B. The smoke dilutions were 1 to 5.4, 1 to 8.0, 1 to 16.0, and 1 to 32.0.

The cigarette brands A and B were selected on the basis of their tar and nicotine contents. Brand A had a high tar and nicotine content, whereas brand B had a low tar and nicotine content. The cigarettes were equalized in length to 65 mm. and climatized at 60% relative humidity in a descicator for at least several days until immediately prior to their actual use in the smoking machine.

The next slide shows the experimental layout of a single two-days' experiment on 72 mice. On the first day, the experiment started with determining a first and second salivation measurement on 36 mice. These mice were then rankordered according to salivary performance and systematically assigned to any one of six treatment groups. The various treatments, applied immediately prior to the third salivation, are thus

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done on very equal groups of mice. The second day's experiment on different mice was done the same, except for different smoke dilutions. Such a two-day experiment was repeated four times. Thus this study includes approximately 72 x 4 x 5 = 1440 salivation measurements.

The next slide presents a layout of the entire salivation experiment. It is noteworthy that any smoke exposures can be compared validly with two types of controls, namely, first with "no exposure" and "machine exposure only," but second also with their own salivary performances during the first and second measurements, as earlier results had shown that non-significant differences in salivation exist during the day. The two types of controls provide further an internal experimental tool to establish justification for pooling the data of single days into larger units for statistical analysis.

Statistical analysis of the data was done primarily using the analysis of variance technique.

In the acute toxicity experiments, groups of six mice were exposed to a 1 to 4.0 or a 1 to 8.0 dilution of whole smoke from either brand A or brand B. Such an experiment was repeated 4 times so that each treatment was applied to 24 mice. The exposures were continued until the mice died, with a maximal exposure time of 60 minutes. Toxicity was determined in terms of percentage of animals dead and by exposure time till death.

The next slide presents data on acute toxicity. The results show 100% lethality with the 1 to 4.0 dilution of whole smokes in each brand. Differential toxicity between brands was, however, found on the basis of exposure time. Death occurred faster with brand A than with brand B, 9.6 vs. 14.5 minutes.

The next slide shows that this effect is highly significant statistically. An F-value of 22.6, whereas only 4.1 is necessary at the 5% level of probability. No deaths occurred with the 1 to 8.0 dilution of either brand. The 60 minute cut-off might have prevented us from obtaining substantiating experimental evidence.

We can, however, conclude that acute toxicity of tobacco smoke depends, as expected, on smoke dilution and brand, and that brand A is more toxic than brand B.

Proceeding now with the results on salivary inhibition, one needs first to establish the validity of pooling the various single day's data before one can draw any valid conclusions from the entire experiment. This can be done in retrospect through comparing the base

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salivation levels of the mice already used for the various treatment groups. The second salivation measurement could well serve this purpose. In statistical terms, the variance due to "brand," "concentration," and the "brand x concentration" interaction should for the second measurement be negligible.

The next slide shows an analysis of variance on the salivary data from the second measurement. Note the very small calculated F-values in "brand," "concentration," and "b x c interaction." This indicates that the experimental animals were very well assigned on the basis of salivation level among the various treatments, and that treatment comparisons from the entire experiment can now validly be made.

The next slide shows the salivary performance of mice upon exposure to various dilutions of whole smoke from brand A. The abcisca represents the time from exposure, in hours, and the ordinate the level of salivary performance, expressed in mm. of boundary displacement. Note the approximate constancy over time and the non-significant differences between the two controls. These curves represent the salivary performances with the 1 to 32, the 1 to 16, the 1 to 8.0, and the 1 to 5.4 dilutions of whole smokes from brand A. Obviously a very dramatic salivary inhibition which is significantly more pronounced in size as well as duration, with higher smoke concentrations.

The next slide shows the salivary performance of mice upon exposure to whole smokes of brand B. It shows the same pattern as we saw with brand A.

The next slide shows the salivary inhibitions of brands A and B at each of the four smoke dilutions. Whereas any differential salivation inhibitory action between brands A and B is not noticeable at the 1 to 32 and 1 to 16 dilutions, a clear distinction can be made with the 1 to 8 and 1 to 5.4 dilutions. This shows that a minimal smoke concentration is required in order to start differentiating the two brands on the basis of salivary inhibition.

Having established the phenomenon that whole smokes exert in inhibitory effect on the salivation in mice, and having found that this salivary inhibition depends on brand and smoke concentration, it becomes then of interest to make optimal the experimental conditions to show this inhibitory effect. Thus we posed the questions: "Do any salivation inhibitory effects due to "brand" and "concentration" show up more pronounced immediately after or one hour after smoke exposure?" and "Do these "brand" and "concentration" effects show up better with "high" than with "low" salivating individuals within the same strain of mice?" These two questions

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were tested on the existing set of data. The experimental mice within each treatment group were separated into "high" and "low" salivators, and statistical analysis with respect to "brand" and "concentration" was performed on "high" and "low" salivating mice, immediately after and one hour after smoke exposure. The size of the F-values for "brand" and "concentration" will determine the best combination of experimental conditions.

The next slide shows a table with calculated F-values under each of the four experimental conditions. Note first of all, that the concentration effect is much more important to salivary inhibition than the effect of brand. This indicates, among others, that for valid brand comparisons the smoke concentrations have to be standardized rigidly. Note further that highly significant "concentration" effects can be detected under any of the four experimental conditions, but in particular so, when high-salivators are used. On the other hand, any differences between brands were totally undetectable immediately after exposure with high-salivating mice, but they do approach statistical significance one hour after exposure with high-salivating mice.

These results indicate that the salivary inhibitions of high-salivating individuals of a high-salivating strain of mice measured one hour after exposure is optimal to measure any differences in the effects of whole smoke concentrations and brands.

In summary:

A new salivation method in mice was employed to study the effects of various dilutions of whole smokes from two brands of cigarettes. Whole cigarette smokes exert an inhibitory effect on salivation under our experimental conditions, the degree of which is determined by the dose and the brand used. A brand with high acute toxicity showed high salivary inhibition, and a brand with low acute toxicity, low salivary inhibition. Thus a relationship was found between acute toxicity on the one hand and salivary inhibition at pharmacological concentrations on the other hand. Some experimental conditions were examined to detect this relationship most prominently. It is suggested that salivation inhibition by cigarette smoke at pharmacological levels might be predictive for acute toxicity of whole smokes at higher concentrations.

In conclusion:

A relationship in mice exists between inhibition of salivation and acute toxicity of cigarette smoke.

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Experimental Design To Study the Effects of Four Dilutions of Whole Smokes from Two Cigarette Brands on the Salivary Performance of Male C57BL/6J Mice

Treatment	Dil.	No. of mice	Measurement				
			1	2	3	4	5
no machine exp.	--	48					
machine exp.	--	48					
Brand A	1:32.0	24					
" "	1:16.0	24					
" "	1:8.0	24					
" "	1:5.4	24					
Brand B	1:32.0	24					
" "	1:16.0	24					
" "	1:8.0	24					
" "	1:5.4	24					

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Experimental Designs of Two-Day Experiment, To Study the Effects of Four Dilutions of Whole Smokes from Two Cigarette Brands on the Salivary Performance of Male C57BL/6J Mice

Day	Treatment	Dil.	No. of mice	Measurement				
				1	2	3	4	5
I	no machine exp.	--	6					
	machine exp.	--	6					
	Brand A	1:32.0	6					
	" "	1:16.0	6					
	Brand B	1:32.0	6					
	" "	1:16.0	6					
II	no machine exp.	--	6					
	machine exp.	--	6					
	Brand A	1:8.0	6					
	" "	1:5.4	6					
	Brand B	1:8.0	6					
	" "	1:5.4	6					

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THE TOXICITY OF VARIOUS DILUTIONS OF WHOLE SMOKE FROM TWO BRANDS OF CIGARETTES

Smoke Dilution	Acute Toxicity			
	Mortality*		Time (Min.)	
	A	B	A	B
1:4.0	(24) 24	(24) 24	9.6	14.5
1:8.0	(24) 0	(24) 0	--	--
Machine-treated	(24) 0	(24) 0	--	--

* Maximal exposure period is 60 minutes.

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ANALYSIS OF VARIANCE OF EXPOSURE TIMES UNTIL DEATH, WHEN MALE C57Bl/6J MICE ARE TREATED WITH A 1:40 DILUTION OF WHOLE SMOKES FROM TWO CIGARETTE BRANDS

Source	DF	MS	F_{cal}	$F_{95\%}$	$F_{99\%}$
Total	47				
Brand	1	1,131	22.62**	4.09	7.31
Experiment	2	276	5.52**	3.23	5.18
Brand x Experiment	2	27	0.54	3.23	5.18
Error	42	50			

** Significant at the 1% level of probability.

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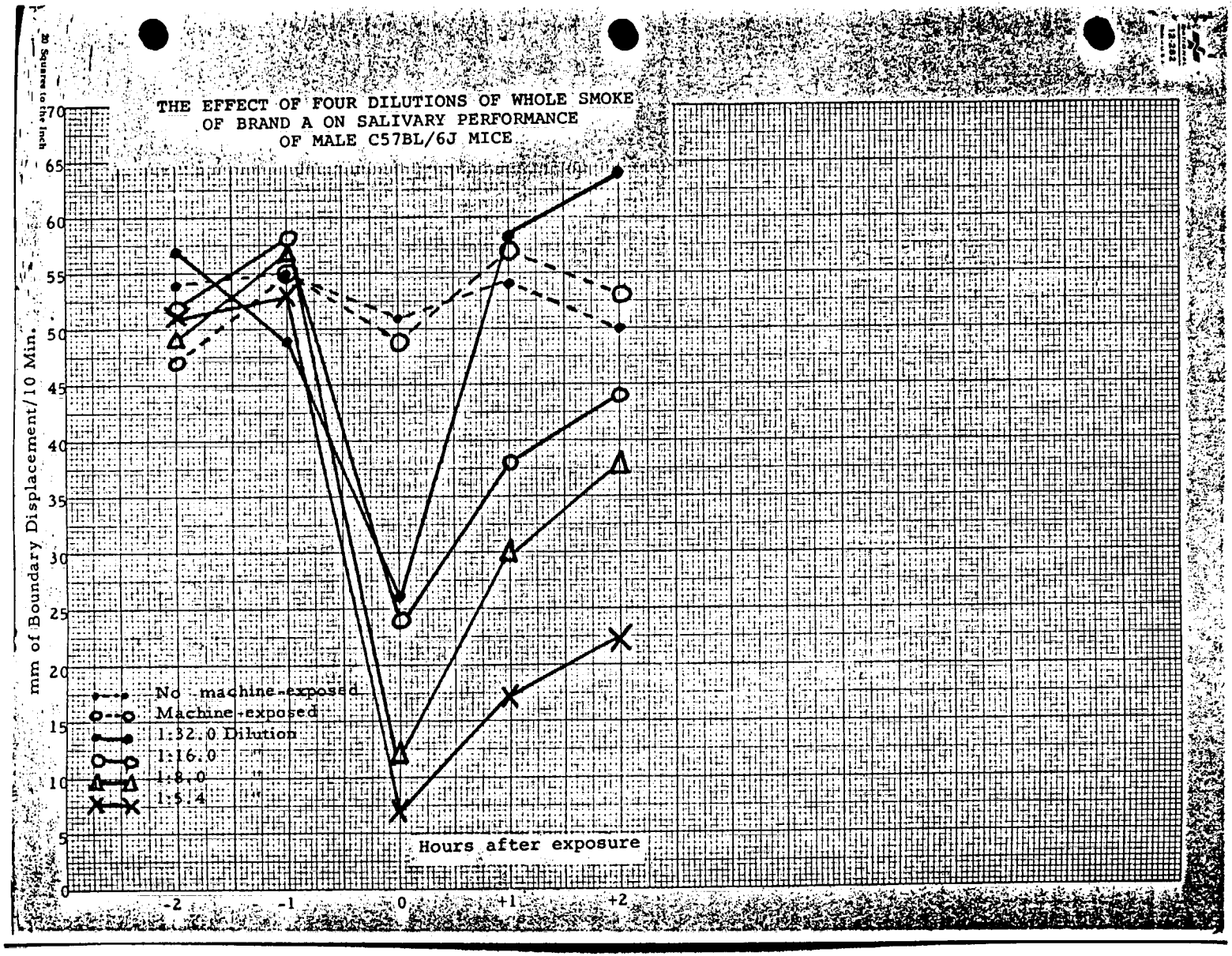
THE EFFECT OF FOUR DILUTIONS OF WHOLE SMOKE
OF BRAND A ON SALIVARY PERFORMANCE
OF MALE C57BL/6J MICE
mm of Boundary Displacement/10 Min.
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
No machine-exposed
Machine-exposed
1:32.0 Dilution
1:16.0
1:8.0
1:5.4
Hours after exposure
-2
-1
0
+1
+2

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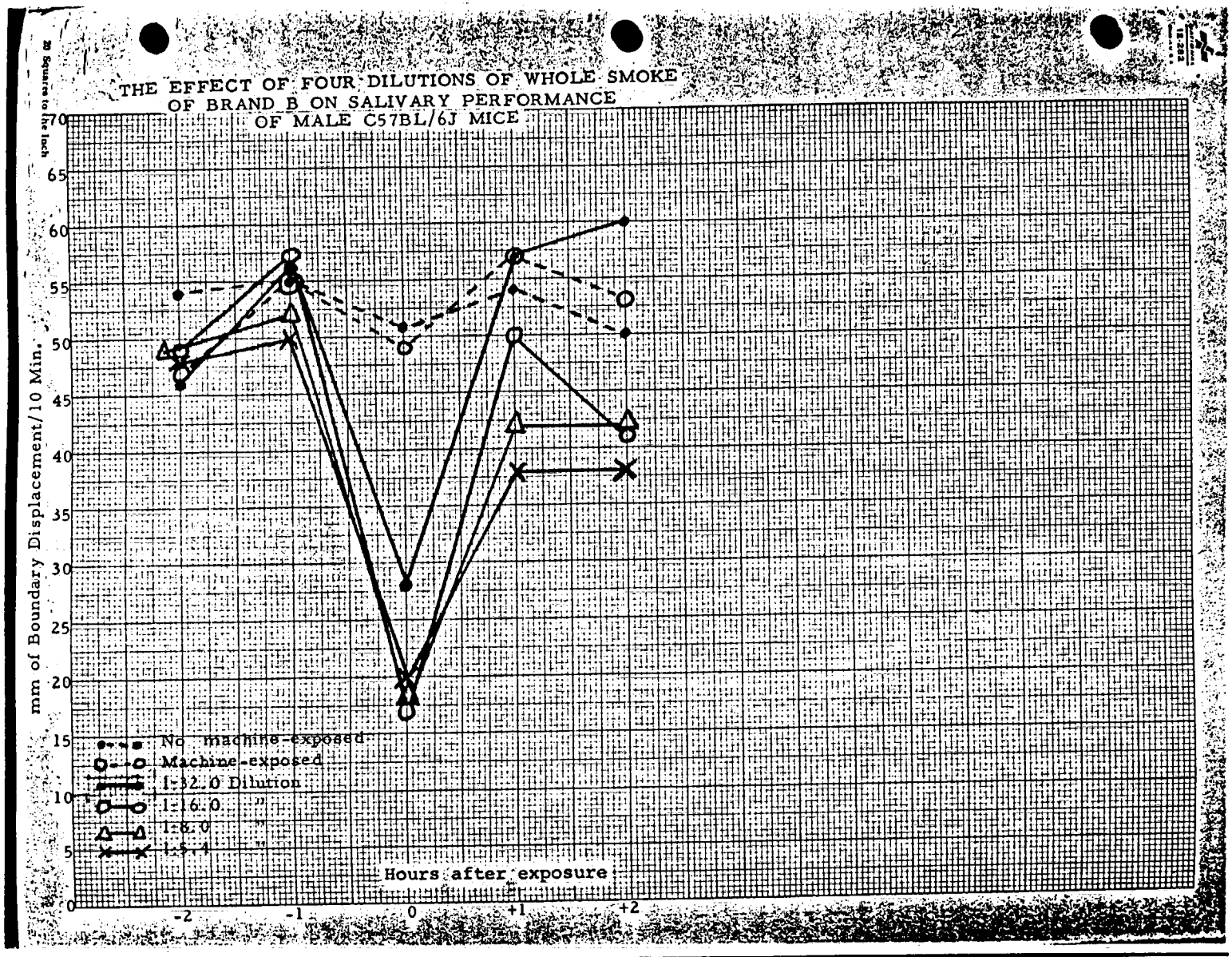
THE EFFECT OF FOUR DILUTIONS OF WHOLE SMOKE
OF BRAND B ON SALIVARY PERFORMANCE
OF MALE C57BL/6J MICE
mm of Boundary Displacement/10 Min.
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
-2
-1
0
+1
+2
Hours after exposure
No machine-exposed
Machine-exposed
1:32.0 Dilution
1:16.0 "
1:8.0 "
1:5.4 "
20 Squares to the Inch

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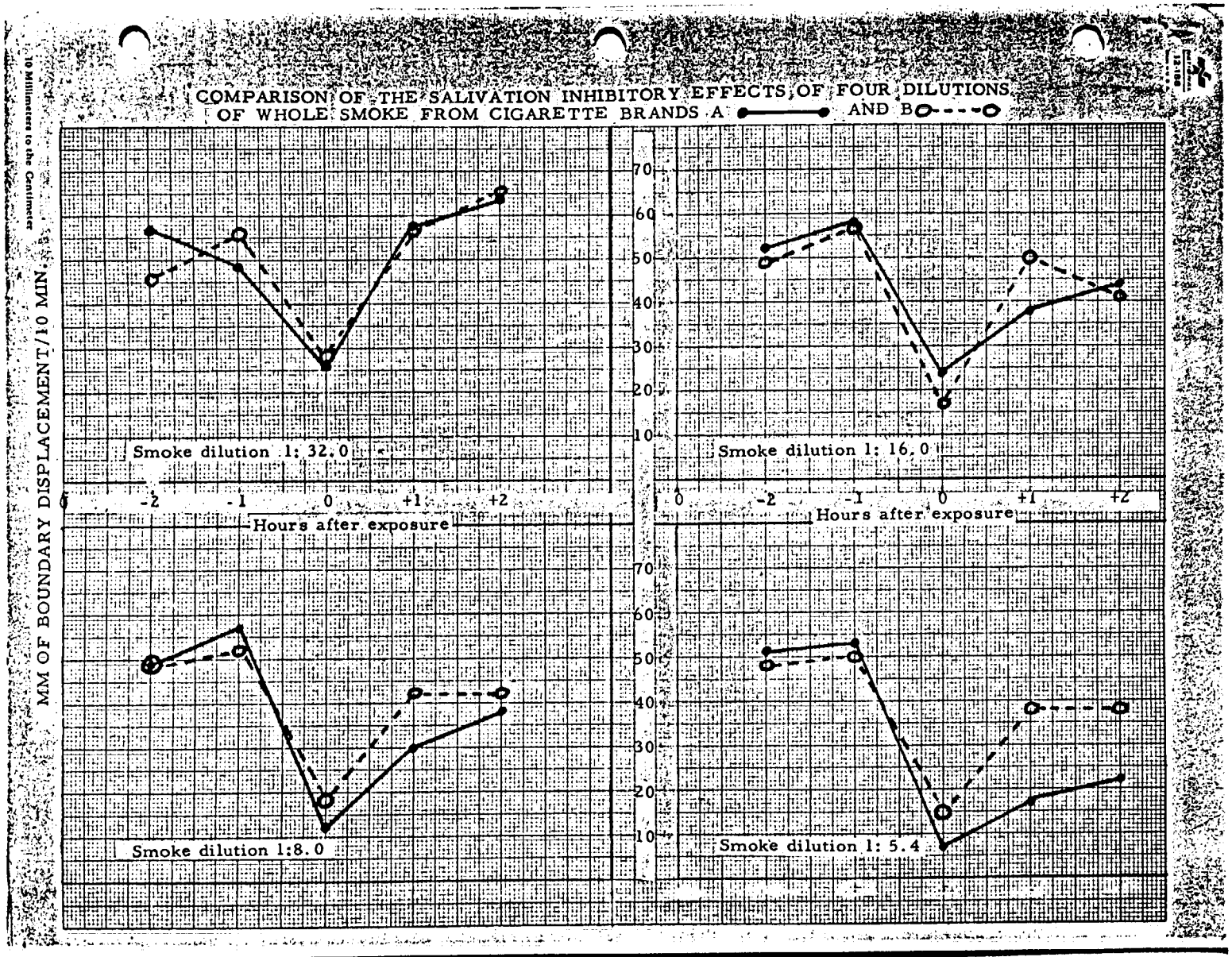

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THE CALCULATED F-VALUES FOR BRAND AND CONCENTRATION, OBTAINED WITH VARIOUS GROUPS OF MICE, AT VARIOUS TIMES AFTER EXPOSURE

Time after exposure (hours)		All		High Sal.		Low Sal.	
		Brand	Conc.	Brand	Conc.	Brand	Conc.
0	F_{cal}	.64	27.30**	1.51	22.82**	0.01	8.95**
1	F_{cal}	3.91*	8.24**	3.64	8.26**	1.11	3.51 **
	$F_{95\%}$	3.84	2.21	3.84	2.21	3.84	2.21

* Significant at the 5% level of probability
** Significant at the 1% level of probability

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